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(71) Applicant *(for all designated States except US)*: NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH–4058 Basel (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HUEBSCHER, Karen [CH/CH]; Stöberstrasse 35, CH–4055 Basel (CH). LEE, Janet [AU/SG]; #13–03 Parkwest Condominium, 4 Jalan Lempeng, Singapore 128794 (SG). FÜRST, Peter [CH/CH]; Cristalinweg 2, CH–4310 Rheinfelden (CH). ROVELLI, Giorgio [CH/CH]; Hofgasse 15, CH–4144 Arlesheim (CH).

(74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent– und Markenabteilung, Lichtstrasse 35, CH–4002 Basel (CH).

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(57) Abstract

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Modulation Of Apoptosis

The present invention relates to methods for modulating the activity of protein-L-isoaspartyl methyltransferase (PIMT) in order to specifically prevent or enhance apoptotic processes in a cell.

Apoptosis or programmed cell death has emerged as a key biological regulatory mechanism which is fundamental to the development and maintenance of tissue homeostasis. Whereas too much cell death can cause degenerative disorders, such as neurodegenerative diseases and neuropathies, too little cell death may lead to proliferative disorders, such as cancer, or autoimmune diseases. Cells undergoing apoptosis generally display shrinkage, loss of cell-cell contact, chromatin condensation, and internucleosomal degradation of DNA. Even though scientists know how to induce apoptosis in different types of cells, the mechanism of this process is still nebulous. There is a need to identify gene expression products which play key roles in the apoptotic process. Such progress is expected to bring about new treatments for those diseases in which apoptosis, or the lack thereof, plays a major role.

Protein-L-isoaspartyl methyltransferase (PIMT), EC 2.1.1.77, is an ubiquitous, mainly cytosolic type II enzyme which catalyzes transfer of the active methyl group of S-adenosyl-L-methionine (AdoMet) onto the α-carboxyl group of atypical L-isoaspartyl sites in peptides and proteins. Isoaspartyl sites are formed by spontaneous intramolecular deamidation or isomerization of Asn-X linkages, wherein Asn is an asparagine residue and X is arbitrary and may be any naturally occurring amino acid. PIMT catalyzes the methylation of unsual D-aspartyl and L-isoaspartyl residues which can accumulate at normal Asn-sites, e.g. in an age-dependent fashion. Methylation of protein carboxyl groups is reversible and leads to an increase in hydrophobicity and the neutralisation of negative charges. It has been suggested that PIMT functions in cells to repair atypical isoaspartate-bearing proteins by converting isoaspartyl to aspartyl sites. Physiological substrates of PIMT include tubulin (J. Najbauer et al., *Biochemistry 35,* 5183-5190 (1996)).

Unexpectedly, we have found that PIMT modulation affects apoptosis. In particular, PIMT induction prevents from apoptosis. Detection of increased levels of expression of PIMT gene products is diagnostic of reduced apoptosis. PIMT is identified as an appropriate

target for therapeutically suitable compounds or signals which interfere with the apoptotic pathway, e.g. compounds or signals preventing or enhancing apoptosis.

Summary of the invention

It is an object of the present invention to provide a method for identifying a compound which modulates apoptosis in a cell.

The invention further relates to PIMT, a functional derivative thereof or a modulator of PIMT activity for use in preventing or alleviating apoptotic cell death in degenerative diseases.

In another embodiment, the invention relates to an agent inhibiting PIMT activity for use in the enhancement of apoptotic cell death in proliferative diseases.

It is yet another object of the present invention to provide a method for diagnosing degenerative or proliferative disorders *in vitro* or *in vivo*.

In a further aspect, the invention relates to kits to determine elevated expression of PIMT mRNA.

Detailed description of the invention

The present invention relates to a method of screening compounds or mixtures of compounds which are potential modulators of PIMT activity comprising (a) providing a test system suitable for determining PIMT activity, (b) exposing the test system to the candidate compound or mixture of compounds; (c) identifying the compound or mixture of compounds which causes modulation of PIMT activity, as measured by the test system.

Within the context of the present invention PIMT relates to protein-L-isoaspartyl methyltransferase having the catalytic activity as described above. The structure and function of PIMT is conserved between a wide variety of species. Preference is given to mammalian, inclusive human, PIMT, in particular murine and human PIMT, which, upon comparison, show a high degree of amino acid sequence homology and similar activity. Examples are human PIMT isoforms I, II and III (GenBank accession nos. D25545, D25546 and D25547, respectively), mouse PIMT (GenBank accession no. M60320) and rat PIMT (GenBank accession no. D11475). Within the context of the present invention a functional derivative of PIMT is a derivative derivable from the respective natural form of PIMT by modification, e.g. by mutagenesis like amino acid substitution, deletion, insertion or addition, or by chemical modification, said derivative substantially showing PIMT activity. A skilled person will be familiar with established techniques both to produce such functional

derivatives and to test for their PIMT activity. Within the context of the present invention, inclusive its particular embodiments, preference is given to the respective natural forms of PIMT.

A suitable test system allows qualitative and/or quantitative determination of PIMT activity. A preferred system is a cellular system containing and expressing endogenous (homologous) and/ or exogenous (heterologous) PIMT encoding nucleic acid. The system enables determination of PIMT gene product level. If desired, such a system also enables monitoring of apoptosis or apoptotic phenomenons. The systems includes suitable controls, advantageously a positive and a negative control.

A compound or signal which modulates PIMT activity particularly refers to a compound which is capable of altering PIMT gene product expression, e.g. a compound or signal which affects PIMT de novo gene transcription and/or PIMT mRNA stabilization, in such a way that the level of PIMT gene product expression is different in the presence of the compound or signal (as compared to the absence of said compound or signal). Modulation of PIMT gene product expression may result in elevated or decreased levels of PIMT gene products. Compounds found to induce an increase in PIMT gene product have an anti-apoptotic effect. Such compounds are capable of protecting cells, e.g. neuronal and glial cells, from apoptosis. If desired, apoptosis is induced in cells before employing these cells in an assay aiming at determining the anti-apoptotic effects of a compound. Suitable methods for inducing apoptosis are known in the art and include e.g. induction of DNA damage, e.g. by irradiation or exposure to mutagenic chemicals, mechanical injury of the cells, growth factor withdrawal, or exposure to low molecular weight non-proteinaceous inducers, such as MPP^+, or to proteinaceous inducers, such as Bax or Bax-related proteins. Compounds found to decrease the amount of PIMT gene product are capable of enhancing apoptosis.

More specifically, the present invention provides a method for identification or design of a compound that is capable of specifically modulating PIMT gene product expression, said method comprising exposing cells expressing biologically active PIMT genes, or a functional equivalent thereof, to at least one compound whose ability to modulate PIMT gene product expression is sought to be investigated, and monitoring the change in PIMT gene product expression level caused by said compound.

Suitable cells or cell lines are readily available and include e.g. neural cells or glial cells, particularly astroglial cells, as well as suitably transformed host cells containing and (over) expressing heterologous PIMT. While DNA encoding PIMT may be expressed in any

suitable host cell, preferred for expression of DNA encoding functional PIMT are eukaryotic expression systems, particularly, mammalian expression systems, including commercially available systems and other systems known to those skilled in the art. Examples of suitable mammalian host cell lines include COS cells. Heterologous DNA may be introduced into host cells by any method known in the art.

In preferred embodiments, PIMT encoding DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express PIMT. The resulting cells can then be produced in quantity for reproducible qualitative and/or quantitative analysis or the effect(s) of potential drugs affecting PIMT function. Vectors usually contain a promoter that is recognised by the host organism and operably linked to PIMT nucleic acid.

Cell-based screening assays can be designed by constructing cell lines in which the expression of a reporter protein, i.e. an easily measurable protein, such as β-galactosidase, chloramphenicol acetyltransferase (CAT), Green Fluorescent Protein, or luciferase, is dependent on PIMT. Such an assay enables the identification or detection of compounds that directly or indirectly modulate PIMT function, e.g. compounds that induce PIMT.

The PIMT gene product that is measured may be PIMT mRNA or PIMT protein. Thus, an elevated level of gene product or elevated gene product expression means an increase in PIMT mRNA production or PIMT protein production and is characteristic of a cell, wherein apoptosis is prevented or alleviated. The increase in PIMT mRNA production or PIMT protein production in an apoptotic cell is determined against a suitable control, i.e. cells suitable for determining PIMT baseline expression. Suitable controls are e.g. "mocktreated" cells, i.e. untreated cells or cells treated with a compound known not to effect PIMT gene product level, in which cells apoptosis has been induced or not. For diagnostic purposes, normal cells for use in determining baseline expression can be obtained e.g. from surrounding cells, i.e. cells surrounding the tissue or cells sought to be diagnosed, e.g. cells surrounding a tumor, from other individuals or from other cell lines. Any increase in PIMT gene product expression can have diagnostic value, but for an anti-apoptotic effect generally the mRNA or protein expression will be elevated at least about twofold. A reduced or decreased level of gene product or reduced gene product expression means a decrease in PIMT mRNA production or PIMT protein production, as compared to suitable controls, and is characteristic of a cell, wherein apoptosis is enhanced.

Methods for detecting mRNA or protein are generally known in the art and include, for example, PCR related techniques, Northern or Western blot analysis, ELISA, RIA, immuno-

precipitation, and the like. Exemplary methods are given in the Examples. The particular method employed is not critical to the practice of the invention.

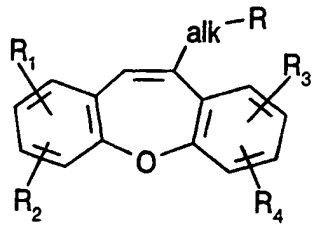
Elevated expression of mRNA may be determined by using a suitable nucleic acid probe. Such probe may comprise ribo- or deoxyribonucleic acids and may contain the entire PIMT coding sequence, preferably the human PIMT coding sequence, a sequence complementary thereto, or fragments thereof. PIMT coding sequences are known from the literature and available from data banks, e.g. GenBank, accession no. D11475 (rat sequence), D25545, D25546, or D25547 (human sequence). Preferred sequences from which to construct probes include 5' and/or 3' coding sequences, sequences involved in intermolecular interaction, and the like. Generally, probes or primers will contain at least about 14 contiguous nucleotides of, or complementary to, the PIMT sequence. Typically, probes are labelled with a suitable label means to facilitate ready detection thereof upon hybridization. Suitable label means include e.g. a fluorescent tag, or a radioisotope, such as ^{32}P. To produce probes and primers, a cDNA molecule containing the PIMT coding sequence may be used. Alternatively, the primers or probes can be synthesized de novo using routine methods. Preferably, the probes will hybridize under stringent conditions.

It has been found that PIMT is induced in gial cells upon treatment with N-(dibenz-[b,f]oxepin-10-ylmethyl-N-methyl-N-prop-2-ynylamine *alias* 10-(N-propargyl-N-methyl-amino)methyldibenz[b,f]oxepine. This low molecular weight compound, related to (-)-deprenyl exhibits anti-apoptotic properties. Such properties may be shown e.g. in vitro in a PC12 cell based assay, in reactive astrocytes or in appropriate in vivo rat models. The effects are dependent on de novo protein synthesis. We identified type II protein carboxyl isoaspartyl methyltransferase (PIMT) mRNA by means of differential display reverse transcriptase PCR to be induced or stabilized upon drug exposure.

Thus the present invention provides a method to exogenously affect PIMT dependent processes in PIMT producing cells. PIMT producing cells, e.g. gial cells or neural cells, can be exposed to a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the PIMT induction response in the presence and absence of test compound, or relating the PIMT induction response of test cells, or control cells (e.g., cells that do not express PIMT) to the presence of the compound. The same strategy can be used to observe the effect of test compounds which modulate the downstream effects of PIMT.

Thus, the invention provides compounds which are modulators of PIMT activity. Preferably, such compounds induce PIMT in neural or glial cells. PIMT-inducing compounds include 10-aminoaliphatyl-dibenz[b,f]oxepines, particularly the oxepines disclosed in Swiss

patent application No. 367/95. Such compounds are 10-aminoaliphatyl-dibenz[b,f]oxepines of formula I



(I),

wherein alk is a divalent aliphatic radical,
R is an amino group that is unsubstituted or mono- or di-substituted by monovalent aliphatic and/or araliphatic radicals or disubstituted by divalent aliphatic radicals, and
R_1, R_2, R_3 and R_4 are each, independently of the others, hydrogen, lower alkyl, lower alkoxy, halogen or trifluoromethyl.

Further classes of compounds may be envisaged, e.g. compounds involving substitutions, side chain alterations and ring modifications of the above-mentioned oxepines. Such further compound may be assayed by measuring the activity thereof in the induction of PIMT synthesis or increasing PIMT actvity.

PIMT activity can be measured by determining the number of methyl groups transferred from S-adenosyl-methioneine to exogenous peptide or endogenous substrates. Briefly, suitable protein substrate samples can be methylated in the presence of PIMT and s-adenosyl-L-[methyl-^{3}H]-methionine. After the reaction is stopped the proteins can be resolved by electrophoresis and conventional staining procedures. For the detection of tritiated proteins by fluorography, gels can be impregnated e.g. with sodium salicylate according to Chamberlain (Chamberlain, J.P., Analytical Biochemistry 98, 132-135 (1979)).

Moreover, the invention is directed to the use of PIMT or a functional derivative thereof as an apoptotic inibitor. In particular, the invention relates to a pharmaceutical composition suitable for the administration to a warm-blooded animal inclusive man, said pharmaceutical composition comprising PIMT protein or a functional derivative thereof in a pharmaceutically effective amount, optionally together with at least one pharmaceutically acceptable carrier and/or excipient. Preferably, such a pharmaceutical composition is used in preventing or alleviating apoptotic cell death in degenerative, preferably neurodegenerative, diseases.

The invention includes a method of screening a compound for an ability to rescue neural or glial cells from apoptosis comprising exposing a neural cell or a glial cell to the compound and observing the levels of PIMT activity, an elevated level being indicative of ability of the compound to rescue said cell from apoptosis. Moreover, the invention provides a composition for rescuing neural cells or glial cells from apoptosis, which composition is effective to increase the activity of PIMT in neural or glial cells.

In a further aspect of the invention, a method is provided for treating a patient suffering from a neural condition comprising administering to the patient a pharmaceutically effective amount of a composition effective to modulate the activity of PIMT in neural or glial cells. Preferably, such a composition is a pharmaceutical composition suitable for the administration to a warm-blooded animal inclusive man comprising an amount of a compound that is effective in the modulation of PIMT activity, optionally together with at least one pharmaceutically effective or acceptable carrier and/or excipient. As set out above, modulation of PIMT activity may be achieved by altering the levels of PIMT expression, or altering the activity of PIMT on its downstream target(s). The effect of therapy set out above will be to delay the onset and/or progress of neural apoptosis associated with neurodegenerative diseases, particularly cerebral ischaemias, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, glaucoma and also general or diabetic peripheral neuropathies.

Moreover, since PIMT is associated with the apoptic process in neural cells, the invention also provides a composition comprising a nucleic acid encoding PIMT or a functional derivative thereof, or a composition comprising an antagonist thereto for use as a medicament in the treatment or diagnosis of neurodegenerative disease. For example, in order to detect any abnormality of endogenous PIMT, genetic screening may be carried out using nucleotide sequences as decribed above as hybridisation probes.

In another embodiment, a transcription unit encoding PIMT or an antagonist thereto is provided for use in a method of treatment of a neural or other condition, for example a condition involving aberrant PIMT expression, by gene therapy techniques. The transcription unit comprises regulatable control regions which include a promoter, together with one or more enhancers and/or locus control region (LCR). The transcription unit may

be delivered to the subject by any suitable means, including viral vectors, especially retroviral vectors, adeno- and adeno-associated viral vectors, non-viral delivery systems, including liposomal and antibody targeted delivery systems, direct uptake of naked DNA and transfer of *ex vivo* transfected cells. The target tissue is advantageously a neural tissue

In still another embodiment, the invention relates to a diagnostic composition comprising a reagent for detecting PIMT gene product. Preferably, the reagent is an anti-PIMT antibody. Such antibody is advantageously provided with means for detecting the antibody, which include enzymatic, fluorescent or radioisotopic means. The antibody and the detection means may be provided for simultaneous, separate or sequential use, in a diagnostic kit intended for the diagnosis of neurodegenerative disorders.

Interference with PIMT expression provides a therapeutic modality. The object is to prevent unwanted apoptosis in a given cell-type. Such therapeutic method is applicable in vivo, in vitro or ex vivo.

In order to enhance apoptosis, PIMT gene product expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to PIMT mRNA, thus preventing transcription. The oligonucleotide may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptidic molecules which specifically inhibit PIMT expression can also be used. PIMT protein can be used as a target in order to identify compounds that enhance apoptosis. Such compounds may be therapeutically useful in proliferative disorders, particularly cancer. Therefore, one aspect of the present invention relates to a compound capable of enhancing apoptosis in a cell, particularly a tumor cell, said compound being identified by a screening method according to the present invention.

More specifically, based on PIMT nucleic acid sequences antisense-type therapeutically active, anti-proliferative agents may be designed. PIMT antisense oligonucleotides and derivatives thereof may be capable of modulating PIMT synthesis in cells. Decreased biosynthesis of PIMT leads to a decrease in PIMT enzyme activity, finally resulting in increased susceptibility to cytostasis and apoptotic cell death . The antisense oligonucleotides and derivatives thereof are therefore appropriate for the prevention and therapeutic treatment of diseases that respond to the inhibition of PIMT synthesis, and thus of PIMT enzyme activity.

Oligonucleotides and their derivatives (as well as salts thereof where salt-forming groups are present) are chosen such that they are specifically hybridizable to DNA or RNA, preferably mRNA, deriving from the gene that encodes functional PIMT, preferably human PIMT. Such an oligonucleotide or oligonucleotide derivative comprises nucleotide units or analogues/derivatives thereof sufficient in number and identity to allow such hybridization. This relationship is commonly denominated as "antisense", and the compounds of the invention are thus PIMT antisense oligonucleotides or their derivatives.

Antisense oligonucleotides and their derivatives specifically bind (hybridize) to the complementary sequences of pre-mRNA or mature mRNA, as defined by Watson-Crick base pairing, interfering with the flow of genetic information from DNA to PIMT protein.

In one preferred embodiment of the invention, the oligonucleotides or their derivatives are specifically hybridizable to the 3' untranslated region of the mRNA coding for PIMT, especially human PIMT. In another preferred embodiment of the invention, the oligonucleotides or oligonucleotide derivatives are specifically hybridizable to the 5' noncoding region of PIMT mRNA. Generally, oligonucleotide derivatives are preferred over oligonucleotides as such.

The term "oligonucleotide derivatives" encompasses tautomeric forms. The term also refers to synthetic species derived from naturally occurring nucleotide subunits or their close homologs and may also refer to moieties which function similarly to naturally occurring oligonucleotides, but which have non-naturally occurring portions, for example at least one building block that differs from the building blocks of a natural oligonucleotide. Thus, oligonucleotides with regard to their backbone may have altered sugar moieties and/or altered inter-sugar linkages, and, with regard to the bases, altered bases may be present. Such oligonucleotide derivatives are best described as being functionally interchangeable with natural oligonucleotides (or synthesized oligonucleotides along natural lines), but having one or more differences from natural structure. All such oligonucleotides are encompassed by this invention provided that they function effectively to show the hybridization properties to DNA or RNA deriving from the PIMT gene, preferably to mRNA.

Hybridization conditions are known in the art and can be found, inter alia, in the reference given below for Northern blotting or in Maniatis et al., "Molecular Cloning - A Laboratory Manual", second edition, Cold Spring Harbor Laboratory Press, 1989, vol. 2, 9.47 to 9.58.

Antisense oligonucleotides or oligonucleotide derivatives according to the invention comprising nucleotide units or analogues/derivatives thereof sufficient in number and

identity to allow hybridization preferably have a length that allows specific binding to the PIMT derived target sequence, especially a length corresponding to 5 to 50 nucleotide units, preferably to 10 to 35 nucleotide units, more preferably to 15 to 22 nucleotide units, and most preferably to 18 to 20 nucleotide units.

In order to allow also for the inclusion of allelic variants of the human PIMT gene and for hybridizable oligonucleotides or oligonucleotide analogues that show minor numbers of mispairing that still allow hybridization, the sequences can vary from those corresponding to the human cDNA by some nucleotides or nucleotide analogues; preferably, up to 3 nucleotides or nucleotide analogues can differ in the sequence of a given oligonucleotide or oligonucleotide derivative with respect to the corresponding PIMT cDNA, more preferably in the sense of conservative mutations.

In yet another embodiment, the invention provides kits to determine elevated expression of PIMT mRNA, e.g. kits containing a nucleotide acid probe as mentioned above, or of PIMT protein, e.g. kits containing anti-PIMT antibodies. The kits may also include auxiliary reagents, such as buffer, enzymes chromogens etc., and/or reaction vessels.

The following Examples serve to illustrate the present invention, but should not be construed as a limitation thereof. The invention particularly relates to the specific embodiments described in these Examples.

Example1: PIMT preparation and activity measurement

PIMT is prepared from cells by collecting the cells and washing them in PBS (10 mM NaPi pH 7.5, 0.1 M NaCl). The cells are resuspended in 4 ml of homogenization buffer (50 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 0.25 M Sucrose, 0.5 mM DTT, 0.5 mM PMSF, 40 mg/ml pepstatine, 40 mg/ml leupeptine, pH8.0). The cell suspension is homogenized by sonication for 30 seconds (s). The suspension, after thawing, is centrifuged at 16'000 g, 4°C for 10 min to pellet the cellular debris. The recovered cytosol is used to measure PIMT activity.

The number of methyl groups transferred from S-adenosyl-methioneine to exogenous peptide or endogenous substrates can be determined as follows. Protein substrate samples (e.g., 7 mM purified tubuline) is methylated for 30 min at 30°C in 0.2 M sodium citrate, pH 6.0 containing 20 ml of cytosol and 50 uM s-adenosyl-L-[methyl-3H]-methioneine (10 - 15 Ci/mmol) in 50 ml. The reaction is stopped by the addition of 50 ml of 0.2 M NaOH, 1% (w/v)

sodium lauryl sulfate. The proteins are resolved by electrophoresis on 0.75 mm thick polyacrylamide gels. The method of Lämmli and Favre (Laemmli, U.K., and Favre, M., 1973, J. Mol. Biol. 80, 575 - 599) is used to run the gels. The gels are stained with Coomassie Blue R-250, destained with 7.5% acetic acid 10% methanol, and equilibrated in water, and dried. For the detection of tritiated proteins by fluorography, gels are impregnated with sodium salicylate according to Chamberlain (Chamberlain, J.P. 1979, Anal. Biochemistry 98, 132-135) prior to drying.

Example 2: N-(Dibenz[b,f]oxepin-10-ylmethyl-N-methyl-N-prop-2-ynylamine

Methyl-propargylamine (4.5 g, 65 mmol) is dissolved in benzene (75 ml) and methanol (25 ml). At 40°C a solution of 10-bromomethyldibenz[b,f]oxepine (7.0 g, 25 mmol) in benzene (25 ml) is added dropwise in the course of half an hour. When the addition is complete, the mixture is stirred for a further half hour at 40-50°C, poured into water, washed three times with water and then extracted with 5 % methanesulfonic acid. The acidic aqueous phase is rendered basic with concentrated ammonia and extracted with diethyl ether. The ethereal phase is dried over sodium sulfate and concentrated by evaporation. Crystallisation of the residue from petroleum ether yields N-(dibenz[b,f]oxepin-10-ylmethyl-N-methyl-N-prop-2-ynylamine *alias* 10-(N-propargyl-N-methyl-amino)methyldibenz[b,f]oxepine (5.3 g, 77%). Melting point: 66-67°C.

Example 3: Protection from apoptosis by PIMT

To elucidate the mechanism of protection from apoptosis differential display reverse transcriptase PCR is used. The response of astroglial cells to N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine is investigated. Emphasis is put on astroglia cells due to increasing evidence supporting the importance of glia in neuronal cell regeneration in the central nervous system and peripheral nervous system. A gene identified to be induced is protein carboxyl isoaspartyl methyltransferase (PIMT). The role of PIMT in protecting from programmed cell death is further addressed by overexpression in COS cells.

Materials

^{33}P-dATP (1000-3000 Ci/mmol) and ^{32}P-dCTP (3000 Ci/mmol) are purchased from Amersham. Superscript II Reverse Transcriptase is purchased from GIBCO/BRL and Pfu Taq DNA Polymerase from Stratagene. Random and oligo-dT primers are supplied by Boehringer Mannheim.

PIMT expression vector: a human PIMT (hPIMT) or a rat PIMT full length cDNA clone is used comprising the DNA sequence available under accession no. D25546 or D11475, respectively, from GenBank. hPIMT or rat PIMT is excised as an *Eco*RI-*Apa*I fragment and subcloned behind the CMV promoter into mammalian expression vector pcDNA3 (Invitrogen).

Animals: Tif:RAlf (SPF) rats are kept with food and water ad libitum and on cycles of 14 h light and 10 h dark.

Primary astroglial cell cultures: Astroglia cultures are routinely grown on 10-cm plastic dishes for tissue culture previously coated overnight at room temperature with a 7 ml solution of 0.25 mg/ml poly-DL-ornithine) in 0.15 M borate buffer pH 8.4. Next morning dishes are rinsed twice with 10 ml of sterile bi-distilled water, allowed to dry in the hood and immediately used or stored up to one week at room temperature. New born rat pups (one to two day old animals) are killed by decapitation. Heads are sprayed with 70 % ethanol, allowed to dry and placed in a sterile hood. Dissections are carried out using standard sterile conditions and performed in calcium/magnesium-free nutrient mixture Ham's F10 medium. Brains are liberated by using small scissors and watchmaker's tweezers. The two cortical hemispheres are separated from the rest of the brain, the meninges pealed off, olfactory bulbs and basal ganglia are removed. The cleaned cortical hemispheres are finely chopped using a scalpel blade and further triturated by passaging through a 5-ml pipette. The equivalent of two cortical hemispheres is plated per 10-cm dish in 10 ml growth medium (DMEM, GIBCO/BRL Cat No 41965-039) supplemented with 10 % heat inactivated horse serum, 10 % calve serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Dishes are incubated for about 5 days at 37°C, with 5 % CO_2 or until cells reach confluency. At this stage, the astroglia monolayer is rinsed once with calcium/magnesium-free PBS and incubated for about 5 min at 37°C with 3 ml of 1x trypsin-EDTA. Trypsin digestion is stopped by adding 3 ml growth medium and cells detached with gentle strokes using a 5 ml pipette. The cell suspension is centrifuged at 3,000 g for 10 min, the supernatant discarded and the cell pellet is resuspended in 1 ml growth medium and gently triturated by passaging 8 times through a Gilson P1000 tip. Large cell aggregates are left to sediment for a few minutes then, the cell suspension is transferred to a fresh tube. The large chunks of tissue are triturated a second time and then the two cell suspensions are combined. Large, un-dissociated cell aggregates are discarded. Cells are split 1:4 in 10 ml fresh growth medium

and seeded to about $1x10^6$ cells per 10-cm dish. Gene induction experiments are performed as soon as the cell monolayer reached confluency.

Gene induction experiments: Control plates are mock treated and left uninjured or are injured by scraping cells off the plate in a criss-cross type fashion with a distance of 0.5 cm between lines using a razor blade. N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine is added to injured and non-injured cells at a concentration of 100 pM or 50 nM for different lenghts of time before isolating total RNA. N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine is readded after 24 h for longer incubation periods without readdition of fresh medium or serum.

Total RNA isolation: For RT-PCR experiments total RNA is extracted using Qiagen's RNeasy Kit (Qiagen No. 74104). Cells are directly lysed in the plates and cell membranes are disrupted either by pipetting the lysate up and down through a 1 ml pipette tip or by using a Quiashredder column (Qiagen No. 79654). For Northern blot hybridisations total RNA is extracted following a single-step RNA isolation procedure described in the manual *Current Protocols* (Teton Data Systems).

Semi-quantitative RT-PCR: 500 ng total RNA is heat denatured for 5min at 70°C together with either a random hexanucleotide primer or a poly(A) primer in a total volume of 11 µl and cooled on ice before reverse transcription is performed using 1 µl (200 U) SuperScriptII RNase H^- Reverse Transcriptase (GibcoBRL) in the presence of 4 µl 1st strand buffer provided with the enzyme, 2 µl dNTPs (10 mM), 2 µl DTT (0.1 M), 1 µl RNasin (Stratagene) at 42 °C for 1 h. The reaction is then incubated for 10 minutes at 55 °C with RNaseH. The cDNA concentration is measured in a spectrophotometer and an equal amount of each sample is used for PCR amplification. A primer pair specific for rat PIMT (5'-TAATCCA-CCAACCTCCGCAAG-3' (SEQ ID NO 1) and 5'-CAGGACCGACTGGCAATATC-3' (SEQ ID NO 2)) is designed and as a control GAPDH is amplified using primers 5'-CTACATGTTCCAGTATGACTC-3' (SEQ ID NO 3), and 5'-AGCCTTCTCCATGGTGGTG-3' (SEQ ID NO 4). The PCR mix is a multiple of: 10 µl PCR buffer provided, 1 µl dNTPs (5 mM each), 82 µl sterile H_2O, 2 µl of each primer (20 µM) and 2 - 4 µl cDNA and 1 µl *Pfu* DNA Polymerase (Stratagene) and 1 µl 1:10 diluted ^{33}P-dATP. DNA is denatured at 94 °C for 5 minutes followed by 15 to 35 cycles of amplification (94°C, 30 s; 55°C 30 s; 72°C 60s). After amplification 10 µl is taken off, 2 µl loading buffer is added and of this 3 µl is run on a non-denaturing polyacrylamide gel for 2 to 3 hours (200 V, 50 W). All reactions are perfomed

using filter tips in order to prevent carry-over contamination. Signals are analysed and scanned in a phosphorimager (Molecular Dynamics).

Northern Blots: 2 to 5 µg total RNA is dissolved in DEPC treated H_2O to a volume of 5.5 µl and combined with 1 µl 10xMOPS buffer, 3.5 µl formaldehyde (37 %) and 10 µl formamide, heated for 15 minutes to 65 °C and cooled on ice before adding 2 µl gel loading buffer. The samples are separated on a formaldehyde containing agarose gel (1 %) for 3 hrs at 4 V/cm without recirculating the buffer with a 1 kb DNA ladder as size marker (GibcoBRL) in one lane. After electrophoresis the gel is rinsed several times in DEPC-H_2O and soaked 2 x 15 minutes in 20xSSC before a capillary blot is set up following the manufacturer's recommendations for Hybond N+ membranes (Amersham Life Science, Amersham, UK). The RNA is fixed to the membrane by incubation in 0.05 N sodium hydroxide for 5 minutes at room temperature followed by a brief wash in 2x SSC.

Hybridisations: Prehybridisations are performed by incubating the membranes at 65°C for 4h in hybridisation solution (5x SSC, 5x Denhardt's, 0.5 % SDS) containing 10 µg/ml denatured salmon sperm DNA. After 4h this solution is replaced with hybridisation solution, denatured probe DNA is added and incubated overnight at 65 °C.

For DNA probes vector pcDNA3 containing hPIMT is linearised with *Apa*I, gel-purified and labelled with ^{32}P-dCTP (3000 Ci/mmol) (Amersham, Life Science, Amersham, UK) by random primed labelling. Labelled probe is purified by centrifugation through a CHROMA spin -30 column (Clontech, Palo Alto, CA, USA) and denatured at 95 °C for 10 minutes before adding to 20 ml hybridisation solution.

After overnight incubation membranes are washed 2x 15 min at room temperature in 2x SSC/0.5 % SDS, 15 min at 65 °C in 1x SSC/0.1 % SDS and 15 min at 65 °C in 0.1x SSC/0.1 % SDS.

Cos1 apoptosis assay: Plasmids pcDNA3 (Invitrogen), pcDNA3CAT (Invitrogen).

Transfection and Cytochemistry: The test plasmids, which encode PIMT, Bax, or CAT as well as a neomycin resistance marker and an SV40 origin of replication, can be transfected in an equal ratio together with pcDNA1beta-galactosidase in a vast excess to ensure that most of the transfected cells contain the beta-galactosidase plasmid. As pcDNA1beta-galactosidase does not contain a neomycin gene it will not be selected for unless it contains the test plasmids, however it does contains the SV40 origin of replication ensuring it is replicated along with the test plasmids. 1 µg of DNA is transfected per 5 x 10^4 and 3 x 10^4 COS 1 cells grown in DMEM (GIBCO BRL) without antibiotics, containing 10% fetal calf

serum in a 5% CO_2 atmosphere. The cells are transfected overnight using the calcium phosphate precipitation method of Pharmacia. For 1 µg of DNA (from a Qiagen midi or mini preparation), 25 µl of H_2O is added to 25 µl of solution A (500 mM $CaCl_2$, 100 mM HEPES, pH 6.95 NaOH) mixed well and after 10 min 50 µl of solution B (280 mM NaCl, 50 mM HEPES, 750 µM NaH_2PO_4, 750 µM Na_2HPO_4, pH 6.95 NaOH) is added and vortexed immediately. After 15 min this solution is be added directly to the cells. After 12-16 hours the cells are washed once in DMEM and then DMEM 10% FCS is replaced. To the transfectants in 24 well dishes, G418 (Sigma) should be added to 800 µg/ml.. Beta-galactosidase histochemistry is performed on the cell culture, three days after the control transfectants (i.e., minus test plasmids), no longer contain any live cells. .

Results

Semi-quantitative RT-PCR: Injured and non-injured astroglial cells are mock treated or treated with 50 nM N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine for 1hour, 24 hours or 48 hours for RT-PCR analysis. Semi-quantitative RT-PCR reveal an upregulation of PIMT after 48 hours by injury and treatment with N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine with 25 cycles of amplification. To confirm this result a fresh batch of cells is treated or mock treated as above for 48 hrs before total RNA is extracted. The initial observation is confirmed with two different concentrations of N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine (50 nM and 100 pM), the lower concentration being more effective in in vivo models.

Prolonged incubation of proteins under physiological conditions leads to an accumulation of spontaneous damage and enhances their methylation accepting capacity. Also, maturation of astroglia cells could affect expression of PIMT. In order to investigate this and the relative effect of injury and N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine on RNA levels of PIMT under conditions potentially enhancing its expression P2 astroglia cells are incubated for more than 14 days before treatment. Treatment is started at total confluency after cells have been kept in the same dish without replacement of medium for 1 week. Total RNA is extracted after 24 hours. PCR performed as above also reveals a band after 30 cycles of amplification in non-injured control cells. A series of PCR cycles starting with 15 and going up to 35 amplification, however, indicates that injury and treatment with N-(dibenz[b,f]oxepin-10-ylmethyl-n-methyl-n-prop-2-ynylamine upregulates RNA levels also under these conditions as visualized by a band occurring already after 20 instead of only

after 25 cycles. In all experiments GAPDH-specific primers are used in order to rule out loading errors prior to cDNA synthesis, PCR-amplification or gel electrophoresis.

Cos1 apoptosis assay: Increased PIMT mRNA levels and cell survival correlate. To address the question of whether PIMT can directly inhibit apoptosis, PIMT is overexpressed in the presence of Bax, under conditions where Bax induces significant apoptosis. For this apoptotic inhibitor test a novel transient transfection assay based on the COS1 expression system is developed. An enriched population of high level expressing transfectants are obtained by modifying standard transfection and G418 selection procedures. This procedure eliminates the problems of stable transfections and the insensitivity of transient transfections. Results are obtained within 5-7 days. The test plasmids encode both the protein of interest, in this case PIMT or Bax, and a neomycin resistance gene. To mark the transfected cells, a vast excess of a plasmid encoding beta-galactosidase is included in each transfection. Apoptosis is detected by a loss of blue staining following beta-galactosidase cytochemistry. Microscopic examination reveals blue apoptotic bodies as well as changes in the cystokeleton in Bax transfected cells, demonstrating that death is occuring by apoptosis and not necrosis. When a plasmid encoding PIMT is co-transfected with BAX there is increased cells survival, showing that PIMT functions as an apoptotic inhibitor.

PIMT helps maintain cellular functions after induction of apoptosis in order to prevent cells from pursuing the cell death pathway.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novartis AG

(B) STREET: Schwarzwaldalle 215

(C) CITY: Basel

(E) COUNTRY: SCHWEIZ

(F) POSTAL CODE (ZIP): 4058

(G) TELEPHONE: +41 61 324 11 11

(H) TELEFAX: + 41 61 322 75 32

(ii) TITLE OF INVENTION: Modulation Of Apoptosis

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TAATCCACCA ACCTCCGCAA G 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGGACCGAC TGGCAATATC 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTACATGTTC CAGTATGACT C 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCCTTCTCC ATGGTGGTG 19

Claims

1. A method for screening compounds or mixtures of compounds which are potential modulators of protein-L-isoaspartyl methyltransferase (PIMT) activity comprising (a) providing a test system suitable for determining PIMT activity, (b) exposing the test system to the candidate compound or mixture of compounds; (c) identifying the compound or mixture of compounds which causes modulation of PIMT activity, as measured by the test system.

2. A method according to claim 1 comprising exposing cells expressing biologically active PIMT, or a functional equivalent thereof, to at least one compound whose ability to modulate PIMT gene product expression is sought to be investigated, and monitoring the change in PIMT gene product expression level caused by said compound.

3. Anti-apoptotic compound identified by a method according to claims 1 or 2.

4. Compound capable of enhancing apoptosis in a cell, particularly in a tumor cell, identified by a method according to claims 1 or 2.

5. Method of screening a compound for an ability to rescue neural or glial cells from apoptosis comprising contacting a neural cell or a glial cell with the compound and observing the levels of PIMT activity.

6. Use of PIMT or a functional derivative thereof as an apoptotic inhibitor.

7. Composition comprising a nucleic acid encoding PIMT or a functional derivative thereof, or an antagonist thereof for use in the treatment or diagnosis of a neurodegenerative disease.

8. Diagnostic composition comprising a reagent for detecting PIMT gene product, and one or more suitable auxiliary reagent.

9. A method of modulating PIMT expression comprising contacting tissues or cells containing and expressing the PIMT gene with an oligonucleotide or oligonucleotide derivative comprising 5 to 50 nucleotide units specifically hybridizable with selected DNA or RNA deriving from the PIMT gene.

10. An oligonucleotide or an oligonucleotide derivative, or a salt thereof where salt-forming groups are present, which is specifically hybridizable with RNA deriving from the gene that encodes functional PIMT.

11. An oligonucleotide derivative, or a pharmaceutically active salt therof, according to claim 10 for the therapeutic treatment of a warm-blooded animal, particularly a human.

12. The use of an oligonucleotide derivative, or a pharmaceutically acceptable salt thereof, according to claim 10 for the preparation of a pharmaceutical composition for the treatment of disorders that respond to the modulation of PIMT synthesis.

13. A kit to determine elevated expression of PIMT gene product comprising a nucleotide acid probe or an anti-PIMT antibody, and optionally auxiliary reagents.

14. Composition according to claim 7 which is a pharmaceutical composition suitable for administration to a warm-blooded animal inclusive man comprising an amount of a compound that is effective in the modulation of PIMT activity, together with at least one pharmaceutically effective carrier and/or excipient.

15. Pharmaceutical composition suitable for the administration to a warm-blooded animal inclusive man comprising the PIMT protein or a functional derivative thereof in an amount effective for inhibition of apoptotis in a cell, optionally together with at least one pharmaceutically acceptable carrier and/or excipient.

INTERNATIONAL SEARCH REPORT

Intern. ial Application No
PCT/EP 97/05481

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/48 A61K38/45 A61K31/70 G01N33/573 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 726 265 A (CIBA-GEIGY AG) 14 August 1996 cited in the application see page 5, line 32 - line 43	3,14
A	WO 96 12797 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA.) 2 May 1996 see the whole document	1-15
A	US 5 273 886 A (D. W. ASWAD.) 28 December 1993	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1998	24/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

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INTERNATIONAL SEARCH REPORT

Interna..nal Application No
PCT/EP 97/05481

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 114, no. 17, 20 April 1991 Columbus, Ohio, US; abstract no. 161928, B. A. JOHNSON ET AL.: "Protein L-isoaspartyl methyltransferase in postmortem brains of aged humans." page 560; column 1; XP002032139 see abstract & NEUROBIOL. AGING, vol. 12, no. 1, 1991, pages 19-24, -----	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ..ial Application No
PCT/EP 97/05481

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		CN 1137039 A	04-12-96
		FI 960529 A	09-08-96
		HU 9600272 A	28-05-97
		JP 8259559 A	08-10-96
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		CA 2203265 A	02-05-96
		EP 0796322 A	24-09-97
US 5273886 A	28-12-93	NONE	